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ORIGINAL ARTICLE

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Two cassava promoters related to vascular expression and storage root formation

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Abstract Cassava (*Manihot esculenta* Crantz) storage roots, organs accumulating large amounts of starch, develop from primary roots via secondary growth. The availability of promoters related to storage-root formation is a prerequisite for engineering root traits in cassava. Two cDNAs, c15 and c54, were identified from a storage-root cDNA library of cassava MCol1505 via differential screening. The transcripts of c15 and c54 were detected in storage roots but not in leaves by Northern analysis. Homology analysis of the deduced amino acid sequences showed that C15 is likely to be related to cytochrome P450 proteins, which are involved in the oxidative degradation of various compounds, while C54 may be related to Pt2L4, a cassava glutamic acid-rich protein. The promoter regions of *c15* and *c54* were isolated from the corresponding clones in a cassava genomic library. A 1,465-bp promoter fragment (*p15/1.5*) of *c15* and a 1,081-bp promoter region (*p54/1.0*) of *c54* were translationally fused to the *uidA* reporter gene, and introduced into cassava and *Arabidopsis thaliana* (L.) Heynh. The expression patterns of *p15/1.5::uidA* and *p54/1.0::uidA* in transgenic plants showed that both promoters are predominantly active in phloem, cambium and xylem vessels of vascular tissues from leaves, stems, and root systems. More importantly, strong β-glucuronidase activity was also detected in the starch-rich parenchyma cells of transgenic storage roots. Our results demonstrate that the two promoters are related to vascular expression and secondary growth of storage roots in cassava.

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Abbreviations *CaMV*: cauliflower mosaic virus · *GUS*: β-glucuronidase · *Nos*: nopaline synthase gene · *pfu*: plaque-forming unit · *uidA*: β-glucuronidase gene

Introduction

Cassava is a perennial root crop providing food for more than 600 million people worldwide. It plays an important role in the food security of developing countries, especially in sub-Saharan Africa (Nweke et al. 2002). There are several major problems that limit the use of cassava roots both by subsistence farmers and by industry. For example, the storage roots are rich in starch (70–90% of their dry weight) but deficient in protein and other micronutrients (Cock 1985). Moreover, once harvested, the roots are subject to rapid post-harvest physiological deterioration, which constrains their storage and marketing (Plumbley and Rickard 1991). These problems could be solved by use of biotechnology provided suitable tissue-specific promoters are available. Therefore, isolation of tissue-specific promoters from cassava is a prerequisite for improving root composition and quality by genetic engineering.

So far, the heterologous 35S promoter from cauliflower mosaic virus (CaMV; Odell et al. 1985) has been the most widely used promoter for cassava transformation (Li et al. 1996; Schöpke et al. 1996; González et al. 1998; Zhang et al. 2000a). Other promoters, such as the cassava vein mosaic virus promoter (Verdaguer et al. 1996) and the phenylalanine ammonia-lyase (PAL) promoter (Beeching et al. 2000), have been recently isolated and characterised. Although the CaMV 35S promoter is active in all tissues of transgenic cassava plants, its level of expression is lower in storage roots than in leaves, which may limit its usefulness for over-expressing transgenes in storage roots (our unpublished

data). Therefore, new promoters with strong activity in storage roots are needed for efficient engineering of storage-root traits. Similarly, to target gene expression in other tissues or in a development-associated manner, novel native promoters are required.

Anatomical studies have revealed two essential processes in storage-root initiation and tuberisation in cassava: (i) rapid cambial activity along the root axis with secondary xylem differentiating towards the inside, with starch restricted to ray parenchyma; (ii) differentiation of enlarged storage-parenchyma cells accumulating starch and replacement of normal secondary xylem fibers (Lowe et al. 1982). The outer region of xylem, including the vascular cambium, is believed to be the most important part where cell division, cell growth and starch formation contribute to the growth of the storage roots. Our goal was to isolate promoters that are preferentially active in storage roots.

In this report we describe the isolation of two root-specific genes, *c15* and *c54*, and their corresponding promoter regions. The specificity of the two promoters was confirmed in transgenic cassava and *Arabidopsis* plants harboring the promoter*::uidA* constructs. The results presented here on histochemical localisation of β-glucuronidase (GUS) activity in mature cassava organs suggest that these two promoters are valuable candidates for targeted gene expression for genetic improvement of cassava.

Materials and methods

Plant materials and growth conditions

Cassava (*Manihot esculenta* Crantz) MCol1505 and TMS60444 plants, provided by CIAT (Centro Interancional de Agricultura Tropical, Cali, Columbia) and IITA (International Institute of Tropical Agriculture, Ibadan, Nigeria), were grown in the greenhouse under a 28 °C/25 °C day/night rhythm. Mature leaves and storage roots used for mRNA isolation were harvested from 1-year-old plants. Analysis of transgenic cassava plants was also conducted using 1-year-old plants. Shoot cultures were maintained on CBM [MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose and 2 μM $CuSO_4$, solidified with 0.6% agar, pH 5.8] at 25 °C under a 16-h photoperiod (90 μmol m^{-2} s^{-1}, TRUE-LITE) and subcultured at 4-week intervals. Conditions for cassava embryogenic suspension cells and somatic embryos were as described by Zhang et al. (2000b).

Plants of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia) were grown at 20 °C under a 16 h/8 h photoperiod in a controlled-environmental chamber. Basic operations of *Arabidopsis* plants were as described by Bechtold and Pelletier (1998).

Construction and differential screening of the cassava storage-root cDNA library

Total RNA was isolated from various cassava organs including mature leaves, petioles, primary roots and storage roots according to Reilly et al. (2001). Isolation of mRNA using oligo dT cellulose was performed according to Sambrook et al. (1989). Double-stranded cDNAs with *Eco*RI ends were produced from storage-root mRNA using the Stratagene cDNA-synthesis kit. The cDNAs were ligated to *Eco*RI-digested λ ZAP II arms and packaged into phage particles with Gigapack Gold packaging extracts from Stratagene. Differential screening of the cDNA library was conducted using ^{32}P-labelled mRNA from storage roots and leaves. Both mRNA fractions were reverse-transcribed into RNA/DNA hybrids using oligo dT primers. Hybrids were labelled with [^{32}P]-dATP and used for differential hybridisation of replica filters containing about 150,000 plaque-forming units (pfu) of the storage-root cDNA library.

Construction and screening of a cassava genomic library

High-quality genomic DNA was isolated from somatic cotyledons of cassava cultivar MCol22 using a protocol modified from the CTAB method (Bohl-Zenger et al. 1997). Aliquots of total cassava genomic DNA were partially digested with *Hin*dIII to enrich fragments 10–25 kb in length. Enriched fragments were cut from an agarose gel, eluted by electrophoresis into a dialysis bag (Sambrook et al. 1989) and cloned into the *Hin*dIII site of the binary cosmid pBIC20 (Meyer et al. 1994) under conditions favoring concatamer formation in a 4-μl volume. Recombinant cosmids were packaged in vitro into λ phages using Gigapack II XL packaging extracts from Stratagene following the manufacturer's instructions. *Escherichia coli* strain NM554 was used for transfection. The library was amplified (Sambrook et al. 1989) and aliquots with a titre in the range of 5×10^6 clone-forming units/μl were stored at −80 °C. DNA from randomly picked clones was analysed by digestion with *Hin*dIII to determine the average insert size.

The genomic library was screened as described by Sambrook et al. (1989) using probes of cDNA c15 and c54. Two independent clones carrying the corresponding genes were isolated and sequenced.

DNA sequencing and nucleotide analysis

Sequencing was carried out with the 373 DNA sequencer from Applied Biosystems following the manufacturer's instructions. Sequence analysis was performed using the Wisconsin GCG package (Madison, WI, USA; Devreux et al. 1984). TFSearch (version 1.3) was used for searching putative transcription factor binding sites (Heinemeyer et al. 1998). The program is available at http://www.cbrc.jp/research/db/TFSEARCH.html. PlantCARE (http://intra.psb.ugent.be:8080/PlantCARE/index.html) was used for determining plant *cis*-acting regulatory elements and for promoter sequence analysis (Lescot et al. 2002). Multiple sequence alignment and conserved domain search were performed at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). The secondary structures of proteins were predicted using GOR IV (Network Protein Sequence Analysis; Combet et al. 2000), which is accessible at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html.

Construction of promoter::uidA fusions

A 1,465-bp promoter fragment of *p15*, named *p15/1.5* (accession no. AY217352), and the complete 1,081-bp 5′ region of *p54*, named *p54/1.0* (accession no. AY217353), were produced by polymerase chain (PCR) reaction using oligonucleotide primers containing an *Eco*RI restriction site in forward orientation and an *Nco*I restriction site covering the site of translation initiation. PCR was carried out using the proof-reading Qiagen kit. Amplified products were digested with *Eco*RI and *Nco*I and cloned into a Bluescript derivative that contains a *uidA* reporter gene with a nopaline synthase (Nos) terminator to give rise to vectors pBP15GUS and pBP54GUS. The promoter regions of the new vectors were sequenced to make sure no mutation occurred during the PCR amplification. The CaMV 35S promoter between *Eco*RI and *Nco*I of binary vector pCambia1301 was replaced by *Eco*RI–*Nco*I fragments of pBP15GUS and pPB54GUS to generate new binary vectors pCP15GUS and pCP54GUS, respectively. In these vectors

p15/1.5 and *p54/1.0* are thus translationally linked to *uidA*, which contains a catalase intron to prevent its expression in bacteria. The binary vectors were introduced into *Agrobacterium tumefaciens* strain LBA4404 via electroporation.

Transient and stable transformation

To investigate the activity of the *p15/1.5* and *p54/1.0* promoters, cassava embryogenic suspension cells were bombarded with pBP15GUS and pBP54GUS. A pLS1 (Lacks et al. 1986)-derived plasmid that contains the *uidA* gene driven by the CaMV 35S promoter was used as a positive control. The protocol for biolistic-mediated DNA delivery to cassava suspension was as described by Zhang and Puonti-Kaerlas (2000). The bombarded suspension cells were subcultured on SH medium [SH salts (Schenk and Hildebrandt 1972) with MS vitamins, 12 mg/l picloram and 6% sucrose, pH 5.8] for 48 h before GUS assay.

Transformation of cassava (cultivar TMS60444) was conducted using *Agrobacterium*-mediated gene transfer essentially as described by Zhang et al. (2000b). *Arabidopsis thaliana* (ecotype Colombia) was transformed using *Agrobacterium* vacuum infiltration of flowering plants (Bechtold and Pelletier 1998). Ten *Arabidopsis* plants were transformed for each construct and mature seeds were harvested. Hygromycin-resistant transformants were germinated on MS medium supplemented with hygromycin and transplanted to pots. Two-week-old plants were used for GUS assay.

Southern and Northern analysis

To analyze the number of inserted transgene copies and GUS expression levels of wild-type and transgenic cassava plants, Southern and Northern analyses were performed as described by Zhang et al. (2000b). The insert numbers of *p15/1.5::uidA* and *p54/1.0::uidA* were analysed by digesting the genomic DNA with *Eco*RI, which cuts only once inside the T-DNA of pCP15GUS and pCP54GUS, and by hybridising to the promoter probes and the *uidA* probe.

β-Glucuronidase assays

Histochemical localisation of GUS activity in transgenic plants was carried out essentially as described by Jefferson (1987). Plant material was incubated in a GUS assay buffer [10 mM $Na_2EDTA{\cdot}H_2O$, 0.1% Triton X-100, 0.3% 5-bromo-4-chloro-3-indoldyl β-D-glucuronide (X-Gluc), 0.1 M NaH_2PO_4, 0.5 M $K_3Fe(CN)_6$]. After 16 h incubation at 37 °C, the tissues were washed several times with 96% ethanol to remove chlorophyll and stored in 96% ethanol. After staining and removal of chlorophyll, the tissues were either directly sectioned by hand or embedded in paraffin and then microtome-sectioned.

Results

Identification and expression analysis of c15 and c54

A cassava storage-root cDNA library containing approximately 1.5×10^6 pfu was constructed and used in differential screening by hybridising to radioactively labelled storage-root mRNA and leaf mRNA. By comparison of the hybridisation patterns, potential root-specific cDNAs were identified and purified. From these, two cDNAs, c15 and c54, were sequenced and analysed.

c15 (accession no. AY217351) is 1,840 bp long, including a putative initiator ATG at its 5′ end (+23 base from cDNA start) and a poly(A^+) tail. The deduced amino acid sequence of C15 is shown in Fig. 1a. The protein consists of 511 amino acids and has a predicted molecular weight of 58.6 Da and a calculated pI of 8.3. A sequence-similarity search revealed that C15 protein showed high similarity to cytochrome P450 proteins from other species, such as CYP71E1 of *Sorghum bicolor* (52% identity, 71% positives, accession no. O48958) and CYP71B10 of *A. thaliana* (43% identity, 67% positives, accession no. NP_200536). To further demonstrate that C15 is a member of the P450 proteins, an NCBI conserved-domain search using RPS–BLAST was performed to compare the potential conserved domains of C15 with those of known proteins. The search showed that C15 belongs to the P450 proteins. As the latter share several conserved domains in their primary amino acid sequences, the P450 consensus sequence can be generated by blasting most of the P450 sequences in Protein Blast of NCBI. Figure 1a shows the sequence alignment of C15 with the cytochrome P450 consensus. Several domains that are highly conserved in the primary amino acid sequence of P450 proteins are also present in the C15 sequence, including the proline-rich motif (PPGP), the I-helix involved in oxygen binding ($^{A}/_{G}GX^{E}/_{D}T^{T}/_{S}$), the E-R-R triade (EXXR.....PXRF), and the heme-binding domain (FXXGXRXCXG) (Werck-Reichart et al. 2002). The N terminus of C15 appears to be hydrophilic and has a predicted alpha helix, which may indicate that the protein is anchored in a membrane system. The predicted secondary structure of C15 consists of 29.55% alpha helix, 22.9% extended strand and 47.55% random coil (Fig. 1b). To assess the number of related genes in the cassava genome, genomic DNA was digested with *Eco*RI, *Hin*dIII and *Xba*I, which do not cut within the cDNA sequence, and hybridised to c15 probe. The c15 probe hybridised to two to three DNA fragments (Fig. 1c), suggesting there are two or three homologous genes in cassava. The expression of *c15* could be detected in storage roots, primary roots and petioles, but not in leaves by Northern analysis (Fig. 1d).

The other clone, c54 (accession no. AY217354) is 902 bp long, encoding a predicted protein of 156 amino acids (Fig. 2a). The initiator codon ATG is localised 86 bp from start of the cDNA. The theoretical pI value and molecular weight for the predicted C54 protein are 3.97 and 16.73 Da, respectively. C54 is rich in glutamic acid (31.4%), alanine (16.03%), proline (13.46%), valine (12.82%) and lysine (8.97%). A BLAST search of C54 showed that the highest similarity (60.2%) of C54 is to Pt2L4 (accession no. AY101376, De Souza et al. 2002; Fig. 2a), which is a *Manihot esculenta* glutamic acid-rich protein the expression of which is related to secondary growth of storage roots (Carvalho et al. 2002). The predicted secondary structure of C54 is mostly alpha helix (41.03%) with only 5.77% sheet structure (Fig. 2b). Southern analysis of *Eco*RI-, *Hin*dIII- and *Xba*I-digested genomic DNA samples using the c54 probe showed that there are two or three related genes in

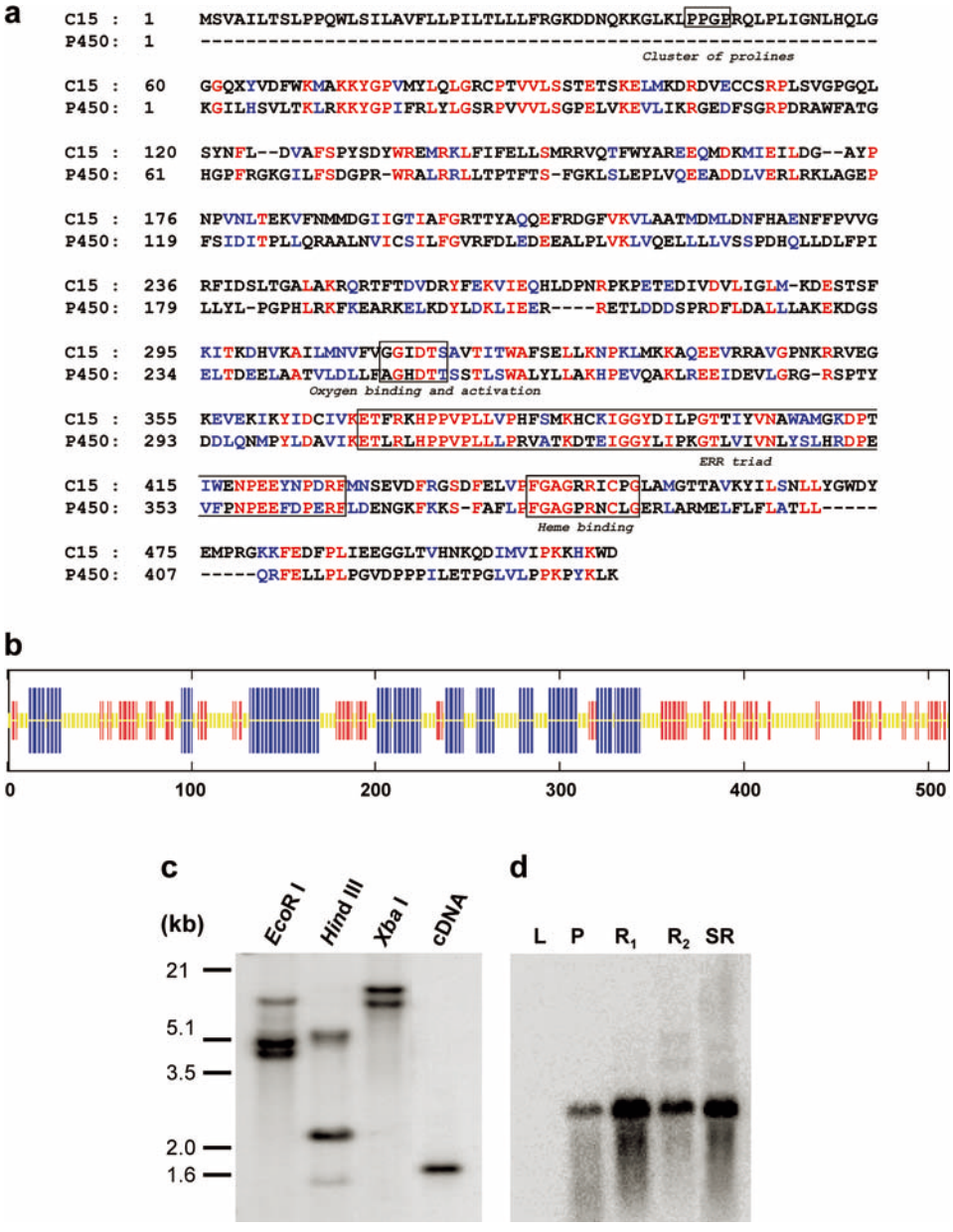

Fig. 1a–d Profile of *c15* from cassava (*Manihot esculenta*) storage roots. **a** Alignment of the deduced amino acid sequence of C15 with that of cytochrome P450 consensus. Identical amino acids are in *red*, similar amino acids in *blue*. Several highly conserved domains in cytochrome P450 proteins are *boxed* with the functions indicated under the boxes. **b** Predicted secondary structure of C15. *Blue bars* alpha helix, *red bars* extended strand, *yellow bars* random coil. **c** Southern analysis of genomic DNA using c15 probe. *Lane cDNA* represents c15 cDNA control. **d** Expression pattern of *c15* in leaves (*L*), petioles (*P*), primary roots of diameter 0–1 mm (R_1) and 1–2 mm (R_2), and storage roots (*SR*)

the cassava genome (Fig. 2c). The expression pattern of *c54* was similar to that of *c15*; in both cases, transcripts were detected in primary roots, storage roots and petioles but not in leaves (Fig. 2d).

Cloning of the c15 and c54 promoters

The cassava genomic library constructed in pBIC20 contains 240,000 independent clones, representing 5.2 genomic equivalents of the cassava genome (750 Mbp per haploid genome). Using radioactively labelled probes of c15 and c54, two corresponding genomic clones of 18 kb and 15 kb were isolated from the genomic library. Fragments containing identical overlaps to the corresponding cDNAs at their 3′ end were subcloned from each clone. Therefore, the upstream sequences of *c15* and *c54* were assumed to contain the promoters *p15* and *p54*, respectively. A 1,465-bp promoter region of *p15*, named *p15/1.5*, and a 1,081-bp promoter region of *p54*, denoted *p54/1.0* (Fig. 3a, b) were analysed using the TFSearch algorithm and PlantCARE database in order to identify putative transcription factor binding sites and conserved plant *cis*-acting regulatory elements.

Sequence analysis of *p15/1.5* showed that a TATA box is localised approximately 60 bp upstream of the start codon ATG (Fig. 3a). There are also several putative CAAT boxes upstream of the TATA box. Interestingly, many matches with 100% similarity to core and matrix regions of known regulatory elements were found as well (Table 1). A search for transcription factor binding sites using the TFSearch algorithm with a threshold score greater than 85 revealed that the

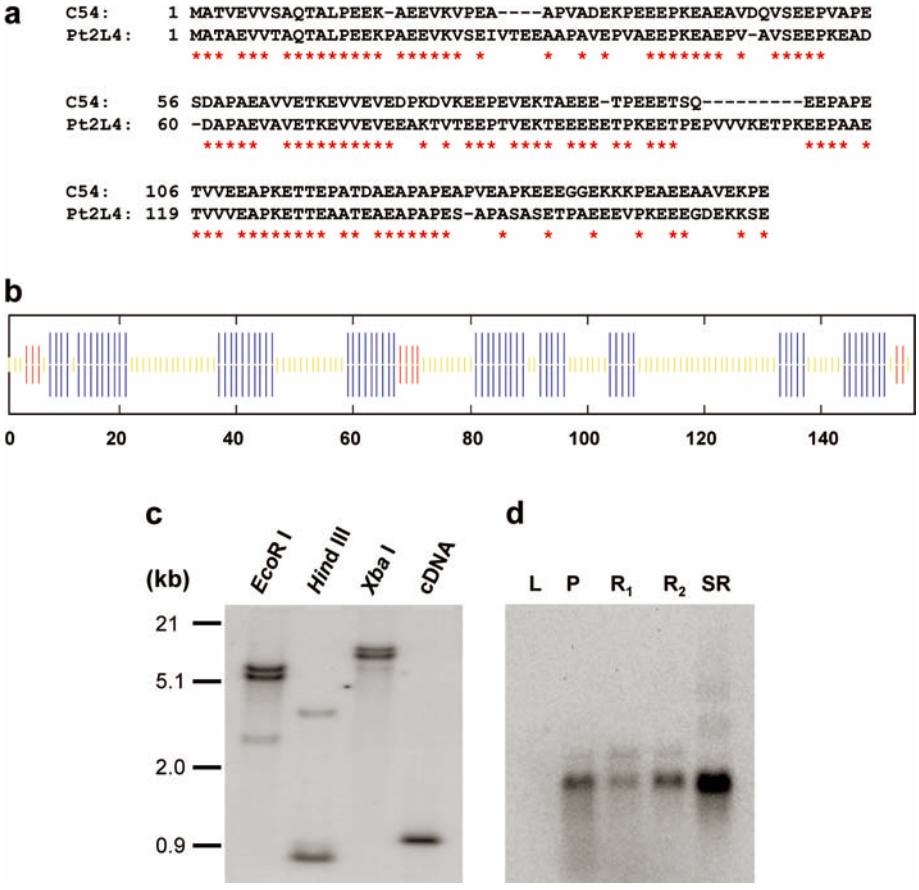

Fig. 2a–d Profile of *c54* from cassava storage roots. **a** Alignment of the deduced amino acid sequence of C54 with that of Pt2L4. Identical amino acids are indicated by *red asterisks* under the alignment. **b** Predicted secondary structure of C54. *Blue bars* alpha helix, *red bars* extended strand, *yellow bars* random coil. **c** Southern analysis of genomic DNA using c54 probe. *Lane cDNA* represents c54 cDNA control. **d** Expression pattern of *c54* in leaves (*L*), petioles (*P*), primary roots of diameter 0–1 mm (R_1) and 1–2 mm (R_2), and storage roots (*SR*)

promoter *p15/1.5* contains the conserved motifs for Athb-1, MYB.Ph, and P binding sites (Fig. 3a).

The TATA box of *p54/1.0* is 117 bp upstream from initiator ATG (Fig. 3b). Besides many putative CAAT boxes, a number of known upstream regulatory elements were also found in this promoter (Table 1). Two conserved motifs for P and SBF1 binding sites were also identified (Fig. 3b).

Transient and stable transformation

To investigate the activities of *p15/1.5* and *p54/1.0*, they were translationally fused to the *uidA* gene to generate constructs pBP15GUS and pBP54GUS, respectively (Fig. 4a). The two constructs were used for transient-expression studies in cassava suspension cells. GUS activity was detected in suspension cells after bombardment with either construct, although the activity is lower than that observed in the controls bombarded with a *CaMV 35S::uidA* construct (data not shown).

To analyse the specificity of *p15/1.5* and *p54/1.0 in planta*, *p15/1.5::uidA* and *p54/1.0::uidA* were introduced into cassava and *Arabidopsis* by *Agrobacterium*-mediated transformation. Eleven independent transgenic cassava plant lines were regenerated from the transformed suspension culture, two lines for *p15/1.5::uidA* and nine lines for *p54/1.0::uidA*. Most of the transgenic plant lines contain one integrated copy of the transgenes (Fig. 4b, c).

GUS expression driven by promoter p15/1.5

Leaves, petioles, young and mature stems, primary roots, secondary roots and storage roots from 1-year-old transgenic cassava plants were collected for GUS assays. Cassava plants containing *CaMV 35S::uidA* were used as a positive control.

Constitutive expression of *CaMV 35S::uidA* was observed in all tissues of cassava leaves, leaf midribs, primary roots and storage roots (Fig. 5a–d). The tissue-specific expression pattern of *p15/1.5::uidA* was confirmed by histochemical localisation of GUS activity in leaves, stems, roots and storage roots of corresponding transgenic cassava plants from the greenhouse (Fig. 5e–m). Intense GUS staining was observed in the vascular tissues of all organs. In the leaves, predominant GUS expression was detected in the vein network, including midribs, major veins and minor veins (Fig. 5e). As shown in the transverse section of midrib (Fig. 5f), preferential expression was found in cells of the endodermis and vascular bundle, but not in the epidermis. In the petioles, strongest GUS expression was confined to the vascular tissues as well, including vascular cambium, phloem, xylem and related parenchyma cells (Fig. 5g).

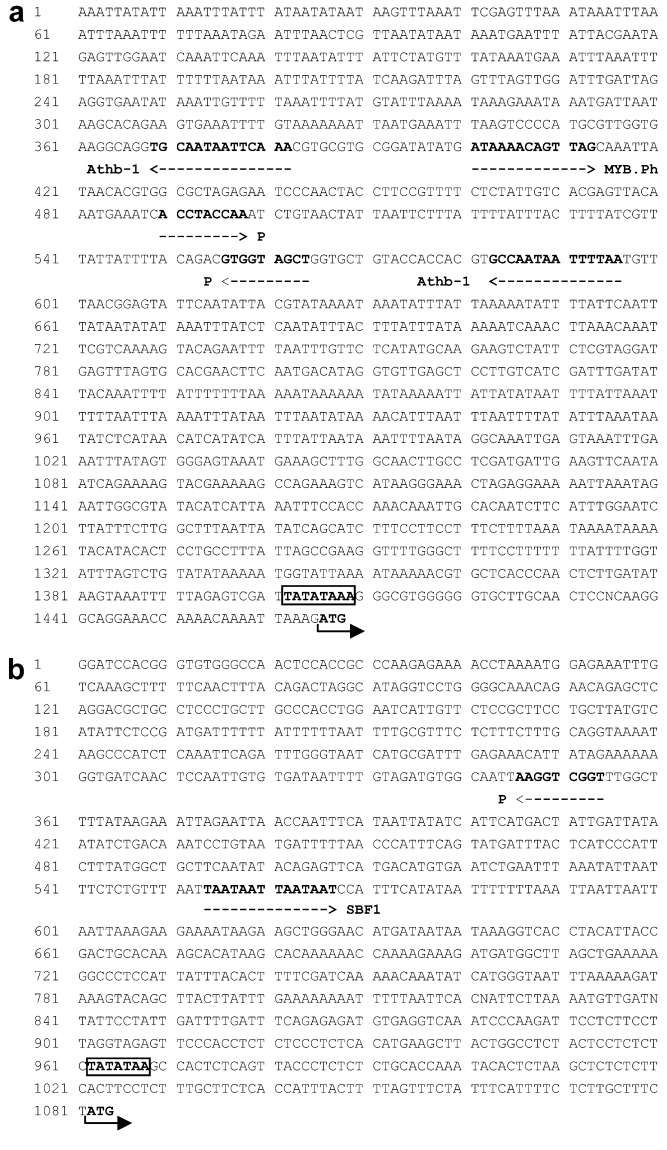

Fig. 3a, b Sequences of promoters *p15/1.5* (**a**) and *p54/1.0* (**b**). *Bold letters underlined with dashed arrows* indicate putative binding sites for transcription factors Athb-1, MYB.Ph, SBF1 and P. The translation start is indicated by a *bold arrow* below the ATG codon on each sequence. The putative TATA boxes are *boxed*

No GUS expression was observed in pith. The expression pattern of *p15/1.5::uidA* in vascular tissues of young and mature stems was virtually unchanged from that observed in the petioles. GUS activity was mainly associated with vascular cambium, differentiating xylem cells, and ray parenchyma, as well as with inner xylem and outer phloem (Fig. 5h, i).

In the primary roots, *p15/1.5* activity was predominant in the vascular cylinder, although relatively low activity was also observed in cortical cells close to the vascular cylinder (Fig. 5j, k). This expression pattern was consistent also in secondary roots, where differentiated xylem is dominant (Fig. 5l). GUS staining was detected in phloem, vascular cambium and xylem. Compared with phloem and xylem, cambium displayed the strongest GUS staining. Strong GUS staining was detected in the storage roots as well (Fig. 5m). After 20 min in GUS buffer, blue staining could be detected in the vascular cambium, and within 1 h, all tissues of the storage roots were blue. Uniform staining was observed in the vascular parenchyma, which is the major site for starch accumulation and storage, and in the external phloem. In storage roots, like in other organs, the vascular cambium consistently showed the strongest activity of *p15/1.5* promoter (Fig. 5m).

To investigate the activity of the promoter in a heterologous plant species, *p15/1.5::uidA* was introduced into *Arabidopsis*. In transgenic *Arabidopsis*, as in cassava, preferential GUS expression was observed in the vascular tissue of leaves, stems and roots (Fig. 6a, b).

GUS expression driven by promoter p54/1.0

Compared with the leaves from *p15/1.5::uidA* transgenic cassava, relatively weak GUS activity was detected in vascular tissues of mature leaves from *p54/1.0::uidA* transgenic cassava plants (Fig. 7a). However, in contrast to *p15/1.5::uidA* plants, GUS staining was also localised to the palisade and spongy mesophyll cells of leaves (Fig. 7b). Strong GUS expression was observed in petioles as well (Fig. 7c, d). Dark-blue staining was detected in the layers of phloem, vascular cambium and xylem. No GUS activity was found in the pith. The expression patterns of young and mature stems were similar to that of the petioles, with strong GUS expression in all kinds of vascular-related tissues (Fig. 7e–g).

Strong GUS activity was also found in the vascular stele of primary roots (Fig. 7h). In the secondary roots, blue staining was present in phloem, vascular cambium and xylem and related parenchyma cells (Fig. 7i, j). Although the levels of GUS expression varied from one line to another, the strongest activity of the promoter was consistently observed in the vascular cambium (Fig. 7d, f, i).

The pattern of GUS staining in the storage roots from seven *p54/1.0::uidA* transgenic cassava lines confirmed that the promoter is active in storage roots (Fig. 7k). Uniform GUS expression was observed in the cambium-derived parenchyma cells of storage roots (Fig. 7l). Similar to *p15/1.5*, the highest *p54/1.0* activity was observed in vascular cambium and xylem vessels. Staining was also observed in phloem (Fig. 7l).

GUS assay of transgenic *Arabidopsis* revealed that the cassava promoter *p54/1.0* was active in heterologous plants as well. The expression of *p54/1.0::uidA* was predominantly confined to the vascular tissues of leaves, stems and roots (Fig. 8a, b).

Discussion

Differential screening of a storage-root cDNA library can be used to identify potential tissue-specific genes, including those specific for roots or leaves. In this study

Table 1 Potential regulatory elements within promoters *p15/1.5* and *p54/1.0* from cassava (*Manihot esculenta*). The matches to known motifs have 100% similarity in both the core sequence and the matrix according to a search of the PlantCARE database. Positions given are relative to the 5′-end of the promoters. The orientation of the motifs is indicated (+, forward; −, reverse). *MeJA* Methyl jasmonate

Promoter	Motifs	No. of motif	Position	Function
P15/1.5	ABRE	1	422 (+)	*Cis*-acting element involved in abscisic acid responsiveness
	AT1-motif	2	184 (+), 622 (−)	Part of a light-responsive module
	CGTCA-motif	1	721 (+)	*Cis*-acting regulatory element involved in MeJA responsiveness
	ERE	1	1015 (−)	Ethylene-responsive element
	G-box	2	423 (+), 469 (−), 577 (+), 1353 (+)	*Cis*-acting regulatory element involved in light responsiveness
	I-box	8	665 (+), 674 (−), 751 (+), 881 (−), 972 (−), 1330 (+), 1375 (+), 1401 (+)	Part of a light-responsive element
	Skn-1-motif	3	801 (−), 824 (+), 1107 (+)	*Cis*-acting regulatory element required for endosperm expression
	TGACG-motif	1	721 (−)	*Cis*-acting regulatory element involved in MeJA responsiveness
	WUN-motif	1	1159 (+)	Wound-responsive element
P54/1.0	AE	1	281 (+)	Part of a module for light response
	Box-W1	1	874 (−)	Fungal elicitor-responsive element
	G-box	1	513 (+)	*Cis*-acting regulatory element involved in light responsiveness
	GA-motif	2	696 (+), 893 (−)	Part of a light-responsive element
	GAG-motif	1	864 (+)	Part of a light-responsive element
	GT1-motif	2	376 (−), 446 (−)	Light-responsive element
	I-box	4	285 (−), 393 (−), 572 (−), 961 (+)	Part of a light-responsive element
	MRE	1	39 (+)	MYB-binding site involved in light responsiveness
	Prolamin-box	2	74 (−), 224 (−)	*Cis*-acting regulatory element associated with GCN4
	Skn-1-motif	3	177 (+), 404 (−), 509 (−)	*Cis*-acting regulatory element required for endosperm expression
	Sp1	1	26 (−)	Light-responsive element
	TCCC-motif	1	919 (+)	Part of a light-responsive element

we characterised cDNAs for two such genes, *c15* and *c54*, as well as their upstream promoter regions.

Transcripts of *c15* and *c54* were detected in the root system but not in leaves (Figs. 1d, 2d). Relatively low levels of expression were also present in petioles compared to storage roots. Similarity searches of the deduced protein sequence of C15 and C54 revealed that C15 is homologous to cytochrome P450 proteins while C54 showed a high similarity to Pt2L4 of cassava. Cytochrome P450 proteins are a superfamily of heme-containing enzymes involved in the oxidative degradation of various compounds, particularly well known for their role in the degradation of environmental toxins and mutagens (Mansuy 1998). The known P450 proteins have highly divergent sequences and only a few conserved domains in their primary amino acid sequence (Gramham and Peterson 1999). The alpha helices of P450 proteins are believed to be essential for the maintenance of their tertiary structure, in which a binding site for a heme cofactor is localised. In C15, several conserved motifs of cytochrome P450 proteins were detected as well, although only 29% of the predicted structure of C15 consists of alpha helix (Fig. 1a, b). The different P450 family members, even when belonging to the same subfamily, have very divergent expression patterns, as shown, for example, in recent studies with *Arabidopsis* (Mizutani et al. 1998). The expression pattern of C15 in cassava is very similar to that of CYP73A5 and CYP83B1 in *Arabidopsis* (Bell-Lelong et al. 1997; Mizutani et al. 1998), all of them being strongly expressed in vascular tissues of leaves, stems and roots. A similar expression pattern was also observed in transgenic *Arabidopsis* containing *p15/1.5::uidA* (Fig. 6). C15 shares the highest similarity with sorghum cytochrome P450 CYP71E1, which catalyses the conversion from Z-oxime to cyanohydrin in the biosynthesis of the L-tyrosine-derived cyanogenic glucoside dhurrin (Jones et al. 1999). Cytochrome P450 genes involved in the synthesis of cyanogenic glucosides have also been cloned for cassava (Andersen et al. 2000), but their sequences identity to C15 on both the DNA and amino acid level is quit low, below 40%. Thus, *c15* appears to be a novel member of the cassava cytochrome P450 proteins with still unknown function.

The C54 homologue Pt2L4 is a glutamic acid-rich protein the expression of which is related to secondary growth of cassava storage roots (Carvalho et al. 2002). The fact that *c54* is expressed more actively in vascular tissues of primary and storage roots than of leaves suggests that *c54* is related to secondary development of cassava roots. This assumption was confirmed by the expression of *uidA* under the control of promoter *p54/1.0*, which was active in phloem and vascular cambium, as well as xylem and related parenchyma cells of

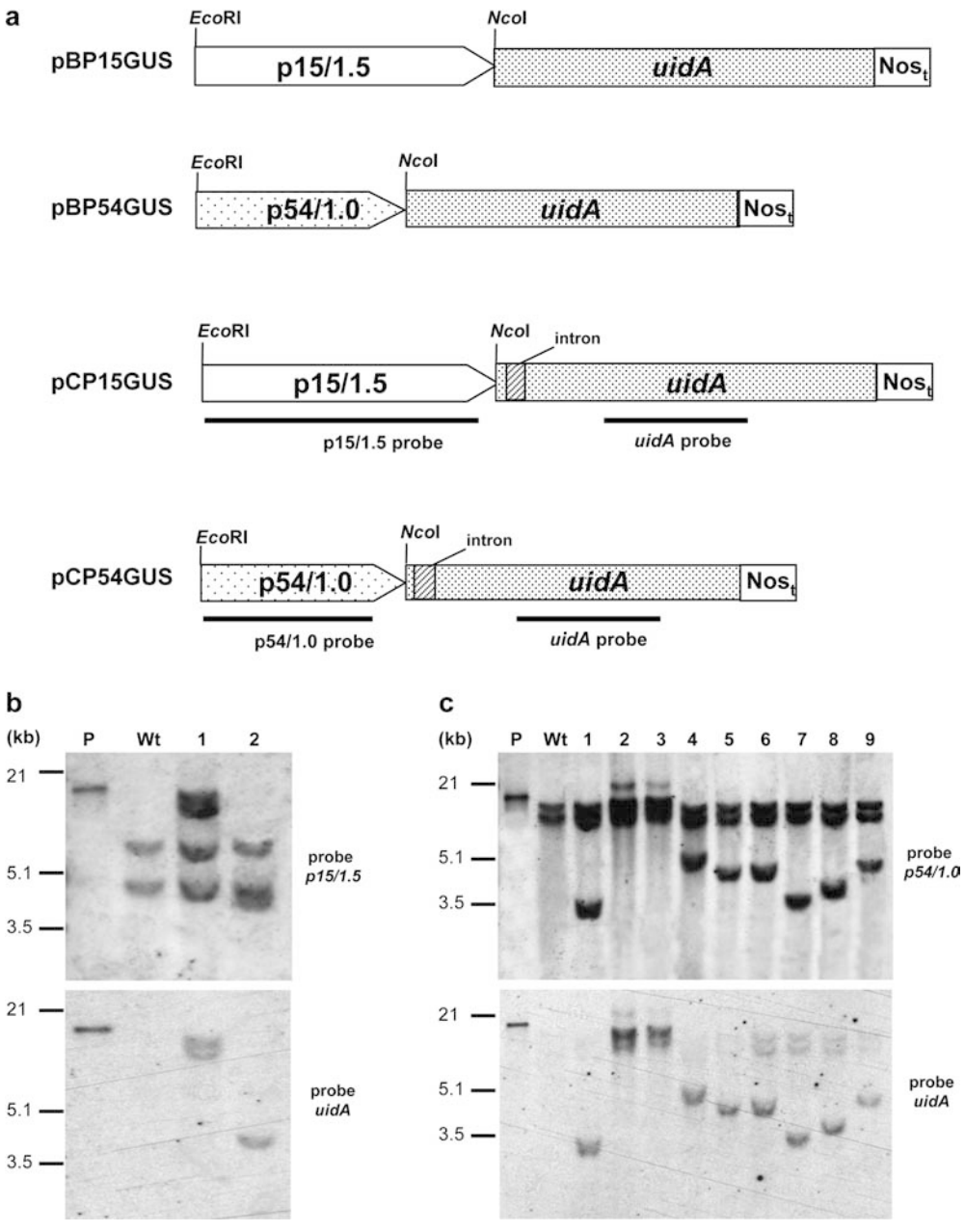

Fig. 4a–c Representation of the *p15/1.5::uidA* and *p54/1.0::uidA* fusions (**a**) and Southern analysis of transgenic cassava plant lines containing *p15/1.5::uidA* (**b**) and *p54/1.0::uidA* (**c**). pBP15GUS and pBP54GUS were used for transient-expression experiments (promoters cloned into a Bluescript derivate, pB). Stable transgenic plants were produced with pCP15GUS and pCP54GUS (promoters cloned into a pCambia 1301 vector, pC), which have a catalase intron in the GUS gene to prevent its expression in bacteria. *Lane P*, plasmid pCP15GUS in (**b**) and pCP54GUS in (**c**); *Wt*, wild-type control; *lanes 1* and *2* of **b** and *1–9* of **c** represent different transgenic cassava lines

primary, secondary and storage roots of cassava (Fig. 7). As the strongest promoter activity was consistently observed in vascular cambium, which plays a key role in the secondary development of storage roots, it can be safely concluded that the activity of *p54/1.0* is related to vascular expression and storage-root formation.

Promoter*::uidA* fusions were used to identify the more precise localisation of *p15/1.5*- and *p54/1.0*-regulated gene expression. Constant GUS expression was predominately detected in vascular tissues, including phloem, vascular cambium and xylem, of transgenic cassava and *Arabidopsis* plants (Figs. 5, 6, 7, 8). Most importantly, intense GUS staining was detected in xylem vessels and xylem parenchyma cells, which are the major site for starch deposition in cassava storage roots. Developmental anatomy of cassava storage roots has shown that the vigorous activity of vascular cambium is responsible for the growth and differentiation of tuberous roots. Therefore, the expression patterns of both *p15/1.5* and *p54/1.0* suggest that the two promoters are indeed related to vascular expression and storage-root formation.

Five and two putative transcription factor binding sites were found on *p15/1.5* and *p54/1.0*, respectively (Fig. 3). Among the transcription factors potentially binding these sites, MYB.Ph (Myb-like protein of *Petunia hybrida*), P (maize activator P of flavonoid biosynthetic genes) and SBF-1 (silencer-binding factor, closely related to GT-1) are involved in flavonoid biosynthesis, a metabolic pathway involving cytochrome P450 proteins (Lawton et al. 1991; Grotewold et al. 1994; Solano et al. 1995). Athb1 is a member of the large homeodomain-Leu zipper protein

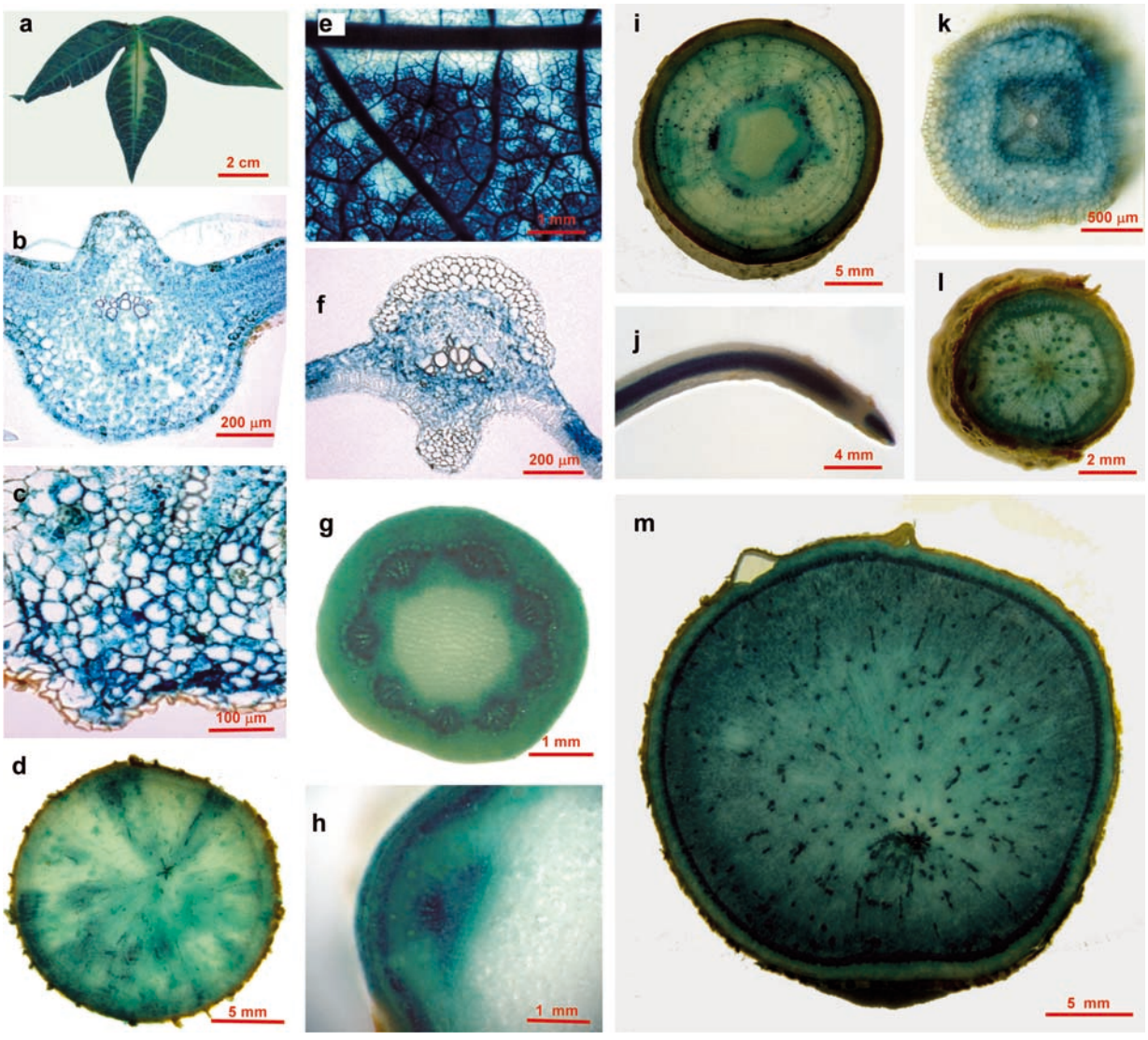

Fig. 5a–m Histochemical localisation of GUS activity in various organs of transgenic cassava. **a–d** GUS activity under the control of the CaMV 35S promoter. Constitutive GUS expression in a leaf (**a**), midrib (**b**), primary root (**c**) and storage root (**d**). **e–m** Expression pattern of *p15/1.5::uidA* in different organs of transgenic cassava: **e** close-up of a stained leaf with strong GUS blue staining in the vein networks; **f** cross-section of the leaf midrib with preferential GUS expression in vascular bundle; **g–i** strongest GUS activity detected in the vascular cambium, phloem and xylem of a petiole (**g**), young stem (**h**) and mature stem (**i**); **j** close-up of a primary root with predominant GUS expression in the vascular stele; **k–m** transverse sections of a primary root (**k**), secondary root (**l**) and storage root (**m**) showing the constant GUS expression in outer phloem, middle vascular cambium and inner xylem

family of *Arabidopsis thaliana* (Sessa et al. 1993), which is required for regulation of root hair development (DiCristina et al. 1996). In addition to the transcription factors, several conserved motifs predicted from the PlantCARE database may be involved in fine-tuning the expression. Although in the current study it is not possible to verify the roles of these transcription factor binding sites or conserved motifs in the promoters, we speculate that they may be co-ordinately involved in the regulation of *c15* and *c54* expression. For example, the multiple copies of the G and I boxes, *cis*-acting regulatory elements involved in light responsiveness, which were localised both in *p15/1.5* and *p54/1.0* (Table 1) may modulate the expression in aboveground organs. Further studies using deletions or truncated versions of the promoters will address this question.

►

Fig. 7a–l Histochemical localisation of GUS activity in various organs of *p54/1.0::uidA* transgenic cassava. **a, b** GUS staining in a leaf (**a**) and transverse section of a leaf (**b**). **c, d** Cross-sections of a petiole (**c**) and petioles from different transgenic lines (**d**) with strong GUS activity in vascular cambium, phloem and xylem; the order of **d** from left to right is wild type and transgenic lines 1–9. **e–g** GUS activity in cross-sections of a young stem (**e**), young stems from different transgenic lines (**f**) and mature stem (**g**); the order in **f** is the same as in **d**. **h** Close-up of a primary root with stronger GUS expression in the vascular stele than in the cortex. **i, j** Transverse sections of secondary roots from different transgenic lines (**i**) and a close-up (**j**); the order in **i** is the same as in **d**. **k** Cross-sections of storage roots from different transgenic lines; the order is the same as in **d**. **l** Close-up of a storage root with GUS expression in the outer phloem, middle vascular cambium and inner xylem

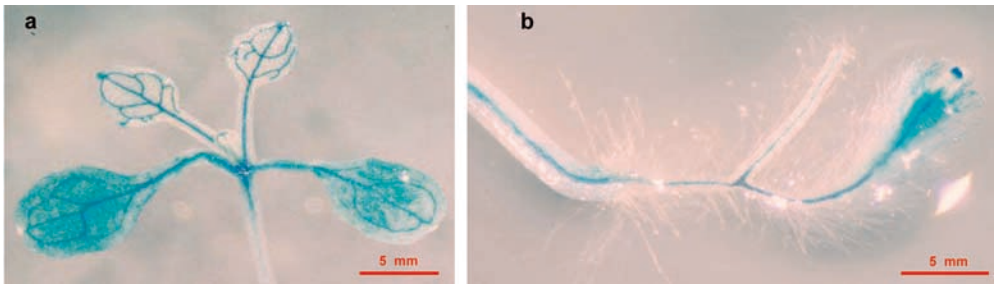

Fig. 6a, b Histochemical localisation of GUS activity in various organs of transgenic *Arabidopsis thaliana*. Expression pattern of *p15/1.5::uidA* in leaves (**a**), petioles (**a**), stems (**a, b**) and roots (**b**)

Although the transcripts of *c15* and *c54* were not detectable in cassava leaves by Northern analysis, the expression of *p15/1.5::uidA* and *p54/1.0::uidA* was observed in corresponding transgenic cassava plants. One reason may be due to the low expression of *c15* and *c54* in leaves or expression related to certain tissues, which cannot be detected by Northern analysis. Using RT–PCR, the expression of *c15* and *c54* could be detected in cassava leaves (data not shown). The promoter of *c15* was strongly active in vascular tissues in the leaves according to GUS expression patterns; therefore, *c15* is possibly only expressed in the vascular tissues of cassava leaves. However, for *c54*, the expression may be weak in leaves.

In cassava biotechnology, organ- or tissue-specific expression of transgenes is of prime importance for targeted genetic improvement. Therefore, the availability of

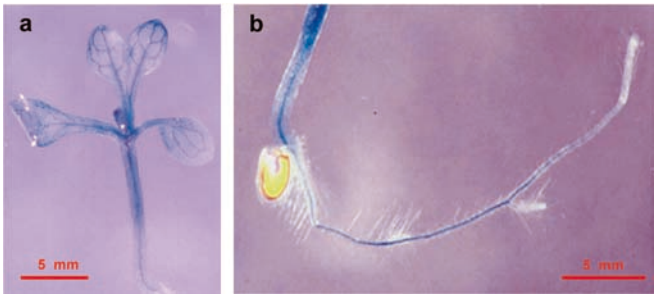

Fig. 8a, b Histochemical localisation of GUS activity in various organs of *p54/1.0::uidA* transgenic *Arabidopsis*. Expression pattern of *p54/1.0::uidA* in leaves (**a**), petioles (**a**), stems (**a, b**) and roots (**b**)

tissue- and organ-specific promoters will be an essential step in this direction. As *p15/1.5* and *p54/1.0* are more active in cassava storage roots than the CaMV 35S promoter, they can be used to express genes in the roots at levels higher than those achieved by using CaMV 35S. Genes of interest include those for improved nutritional value of cassava storage roots, such as genes encoding storage proteins rich in essential amino acids (Zhang et al. 2003). Another potential use could be in the control of post-harvest physiological deterioration (PPD) of cassava storage roots, which is a major constraint for cassava storage and marketing (Plumbley and Rikhard 1991). PPD starts from vascular tissues and xylem strands of the root and spreads to adjacent storage parenchyma. The process is initially observed as a dark-blue vascular streaking developing from wound sites produced by the process of harvesting, followed by discoloration of the storage parenchyma (Noon and Booth 1977). Several PPD-related genes have been cloned in cassava, including catalase (Reilly et al. 2001) and 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (Li et al. 2000). Expressing PPD-related genes under the control of *p15/1.5* or *p54/1.0* may successfully delay the PPD response in cassava.

The *p15/1.5* and *p54/1.0* promoters could also be useful for developing geminivirus-resistant cassava. Geminiviruses are transported through the phloem of vascular tissues during long-distance movement (Hull 2002). Thus, strong expression of anti-viral genes, e.g. RNA inference (RNAi) genes or viral antisense genes, in phloem cells might efficiently inhibit the systemic spread of cassava geminiviruses, such as African cassava mosaic viruses.

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